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FUNGUS CULTURES: CONSERVATION AND TAXONOMIC RESPONSIBILITY

by Emory G. Simmons
Quartermaster Culture Collection
Natick, U.S.A.

Purity, stability, and availability of cultures are major problems of all culture collections. The authenticity of their identifications, particularly as our isolates are pertinent to other scientific disciplines, should be of equally important concern.

The following remarks will (1) enumerate the most widely used methods for the preservation of fungi, their successes and shortcomings, thus serving as a basis for more detailed discussion; (2) summarize a few of the observations and maintenance problems of the primarily taxonomic laboratory with which I am associated; (3) introduce some recently developed methods; and (4) enlarge on my views on the taxonomic and informational responsibilities of a mycological culture collection.

The comments of each of us at a conference on culture collections must be weighted according to the kinds of experience he has had in the field. A brief resume of the U.S. Army's Quartermaster Culture Collection activity therefore is pertinent.

During investigations in the 1940's on the nature of microbiological deterioration of military equipment in the tropics, thousands of fungal and bacterial strains were isolated, identified, and tested for their degradative ability under controlled conditions. These organisms were preserved and became the nucleus of the Quartermaster collection, now believed to be the second largest depository of fungus isolates in the United States. The thousands of strains which have been added in the past fifteen years reflect not only a continuing basic interest in destructive saprophytic microorganisms but also the various and changing lines of research of the microbiologists, enzymologists, taxonomists, chemists, and fungicide and testing specialists who work with them.

Many of our strains are required in the microbiological degradation tests used to determine the acceptability of government purchases. Many others are used in laboratories associated with ours which have a long history of research on the enzymatic degradation of cellulose and related carbohydrates. Our primary concern, then, with physiologic stability centers around maintaining the degradative potentials and certain enzymatic capabilities of our strains.

The QM Culture Collection, like most others, has not been

particularly active in developing new methods of conservation. We have adopted those standard techniques which have met our needs best and which over the years have given us the most satisfactory results, namely, periodic transfer, lyophilization, and oil-covered slants. Of pertinence, however, are some extensive observations we have made on a relatively large number of strains maintained under mineral oil for a minimum of ten years. These data will be discussed below.

Preservation Methods: Used and New

Without doubt, the commonest method of maintaining fungus strains is as living colonies on solidified nutrient media. No organization which is working actively with fungus isolates is immune to the time-consuming labor of transferring and rechecking many of its strains periodically. Large collections which use no other method of conservation face a constant technical burden and the even more serious scientific burden of lost strains and of cultural variation, contamination, and degeneration. Offsetting these problems is the ready availability of the cultures for comparison and transfer.

On the other hand, a collection which maintains essentially all of its strains only in oil-covered slants or in some other type of relatively inactive condition cannot work rapidly or efficiently in identification or biological survey operations. The strains may be fairly well protected, but their utility is limited.

A compromise procedure is used in the QM Culture Collection and in several, but not all, other large collections. Each isolate accessioned at QM is lyophilized if possible; those for any reason unsuitable for lyophilization are stored as oil-covered slants. In the active collection, subject to periodic transfer and immediately available for comparison or distribution, are one or two representative strains of each species or variety, all of the strains derived from type material, all of the strains frequently requested for research or developmental purposes, and all of the strains pertinent to our taxonomic studies. Thus we maintain in active culture 1500 strains taxonomically and physiologically representative of the 9000 QM accessions. The inactive strains of any species are retrieved only when needed for a new area of research, thus minimizing the attention which must be given to large numbers of similar strains.

The biological and technical disadvantages of maintaining cultures in an actively growing condition are grave. It is granted that a very large proportion of any general fungus collection can be carried through the years on a maximum of a half-dozen different media. Thousands of strains are just as stable now as they were when isolated years ago, and this after scores of periodic transfers on one or two of these basic conservation media. Good examples

from our experience are two isolates which have been used very extensively in deterioration studies, Chaetomium globosum QM 459 and a Gliocladium sp. QM 365, widely distributed and used as a "Trichoderma sp. T-1". As nearly as we can determine, these two strains have retained complete physiological and morphological stability throughout 30 and 20 years, respectively, of serial transfers on potato-dextrose and hay-infusion agars. But there is an embarrassing number of species that defy all of our best attempts at stabilization on commonly used media. Fusarium is notorious for its variability and its degeneration from "Normkultur"; many phytopathogens cease to sporulate after their first transfer, thus losing their identifiability.

For ten years my own work has centered around the taxonomy of Alternaria, Curvularia, and Stemphylium. Cultures of a hundred different species in these genera exhibit a discouragingly narrow range of growth variations and color differences. Hence, stable sporulation over a long period of years is essential to the establishment of "representative" strains. Yet many isolates of one of the commonest pathogens, Alternaria solani, become nonsporulating after a very few transfers. It is not enough that I personally am convinced of the identity of such isolates; they are essentially worthless as any sort of taxonomic standard. A similar situation obtains with Curvularia trifolii, often reported to have been isolated. But we never have received a culture under this name that could be reidentified; the donors of such cultures may be confident of their original materials, but I cannot because they no longer sporulate.

Such nonsporulating strains may be usable in some kinds of physiological work. However, a reputable culture collection which distributes them without qualifying statements only invites disaster. A choice must be made between (1) maintaining important nonsporulating material without hope of reidentification and (2) putting some of our efforts into devising means of retaining or inducing sporulation in such strains before the character is permanently lost.

With the dark-spored genera mentioned above, diurnal light and dark fluctuations are sufficient to induce sporulation in some recalcitrant strains. Direct strong light, even sunlight, may be required for others; and the extreme of direct ultra-violet irradiation is a decisive factor for yet others. Some attention has been given to producing abundant conidia in Alternaria solani by means of macerating liquid shake-culture pellets and then incubating them overnight on filter paper (13). These treatments seem drastic and troublesome as maintenance procedures and, in the case of U-V irradiation, may result in mutations; but these or other biophysical shock methods must be used if we are to maintain recognizable, representative strains of many species.

Two other phenomena observed in isolates of these three

genera of Fungi Imperfecti and related to their maintenance as active cultures should be noted, particularly as it is doubted that these phenomena are unique to these genera. All three genera characteristically produce relatively large, dark-walled hyphae and multicelled conidia. When an isolate of any one of them is put through a series of single-spore reisolations for stabilization purposes, it often happens that the mycelium gradually becomes wet, collapsing, and pale; sporulation decreases or ceases. The culture in this condition bears little resemblance to the original isolate. It has been our suspicion that viruses may be the cause of such a decline and that viral infections of fungi may in fact be rather common. Should this prove true, we would have the explanation if not the immediate solution for one of our conservation problems.

The second phenomenon, more readily substantiated because it is observable under microscopic examination, is continued aberrant growth through many single-spore reisolations without decline in vigor of the mycelium or of sporulation. It is possible to demonstrate very often that the hyphae (commonly 5-10μ in diameter) carry a very delicate internal hyphal parasite about 2μ in diameter. With dilute stains these fine hyphae can be seen not only within the host mycelium but also as minute branches extending through the host cell walls and growing out through pores of the conidiophores and basal scars of the conidia. They are of an essentially different magnitude, color, and degree of fragility than those of the host's branches or germination hyphae. We have never been successful in establishing cultures from hyphal tips of these internal parasites.

It is asserted, then, that in addition to variation exhibited in genetically unstable but otherwise truly pure cultures, we are faced with the necessity of combating variation and instability incited by internal fungal and possibly viral parasites. Neither of these latter areas of variability has been studied in any degree; much basic work in them is needed and should be both enlightening and profitable.

The QM active collection is transferred twice each year; the cultures are maintained in refrigerators at about 5°C between the semiannual transfers. Remarkably few strains are lost through such handling. Commonly we must retrieve 50-75 strains from lyophilized or oil-covered preparations after each full transfer of cultures. The strains which fail to grow at any given transfer seldom are the same ones year after year.

For active maintenance of each culture we normally use the same medium indefinitely after it has been chosen. The choice is made after reisolation and study of each new strain on several different media; the conservation medium chosen is the one on which the isolate is culturally most stable and best sporulating. Czapek and malt agars are used for most of the Aspergillus, Penicillium,

and closely related strains. Potato-dextrose agar still is used for a considerable portion of our cultures which have remained stable on this relatively rich medium. Gradually we have been shifting great numbers of isolates, particularly new ones, to media in which the only nutrients are plant juices or decoctions. "V-8 juice" (Campbell Soup Co.) is an excellent substrate for a wide range of saprophytes and plant pathogens such as those found in Stemphylium and Curvularia. Growth is rapid and abundant on 20% V-8 agar (16), and sporulation often is phenomenal. Potato-carrot agar (12; 5), based on a very weak decoction of these two vegetables, gradually is being used to replace PDA for strains which find this latter medium too rich for good sporulation.

One other medium, hay-infusion agar (21), deserves special mention and recommendation, both for isolation work and for conservation procedures. Hay agar is based on a weak decoction of dead, partially decomposed roadside grasses. The medium is completely undefined in a chemical sense except that it is used at pH 6.0-6.5. Its most helpful characteristic is that it does not support the development of masses of mycelium which often interfere with isolation work but that it does permit the abundant and typical sporulation of great numbers of both pathogenic and saprophytic molds. It has proved most helpful in isolation of dark-spored Hyphomycetes, particularly Alternaria, which tend to be heavily mycelial on any medium with added sugars; it is the conservation medium of choice for any Alternaria or Curvularia which will sporulate on it. This same characteristic of suppressing mycelium in favor of sporulation is particularly welcome in the lyophilization procedure where large numbers of spores and relatively little mycelium are desired.

The value of lyophilization for conservation of fungus strains is best documented by the series of reports issued since 1945 (17; 7; 14; 8) by mycologists of the U.S.D.A. Northern Regional Research Laboratory. NRRL pioneered this technique for fungi in 1942 and has firmly established its utility on the basis of intensive experience. Most of the large public and industrial collections in the U.S. have adopted lyophilization procedures, QM having done so in 1944. The observations of all these laboratories are roughly the same, namely, that well over 90 per cent of fungus strains which produce spores can be preserved in lyophil for periods of at least 10-15 years; the viability expectation is much greater than this.

It is a common observation that lyophilized cultures retain their morphological characteristics at least as well as the same strain maintained by periodic transfers. Our laboratory and others (8) have observed that many isolates retrieved from lyophil are morphologically more stable and sporulate more profusely than the

same strains which have been carried actively on nutrient media. In particular, isolates which show a tendency to have nonsporulating patches occasionally stabilize quite well and sporulate vigorously when retrieved from lyophil. It is believed that the mycelial patches are genetically variant and that, because they are nonsporulating, they are eliminated under the rigors of the lyophilization process.

One area of failure with the lyophilization technique deserves special mention and further attention. Neither NRRL (8) nor QM has had any degree of success in maintaining species of Entomophthorales in lyophil. Although some strains, particularly one of Delacroixia coronata (QM 6844, NRRL 1912), produce spores abundantly, only a very poor degree of viability has been achieved after freeze-drying. It has not yet proved possible to extend even this minimal viability for longer than five years, most preparations proving to be nonviable immediately after processing.

References to the retention of physiological characteristics by lyophilized fungi are meager. Our own observations on the retention of cellulolytic activity reveal that this character has not changed appreciably in fungus strains which were culturally stable when lyophilized. Dorothy I. Fennell (6) has made the pertinent remark that "the widespread use of the lyophil method of preservation by industrial laboratories, where vast and expensive fermentations depend on the stability of their cultures, seems to offer overwhelming evidence that physiological characters are maintained unchanged by the great majority of lyophilized cultures."

Preservation of cultures for relatively long periods of time under a layer of sterile mineral oil is a method which has many users but few advocates if any other means of maintenance can be applied. The technique is used routinely for the storage of large numbers of Basidiomycete cultures. The Division of Forest Products, C.S.I.R.O., Melbourne, maintains its collection of 1400 strains in this manner with subculturing every three years. They report better than 99 percent viability with no detectable change in physiologic characteristics, although some of the same strains maintained as active cultures have lost their ability to decay wood (18). The major application of this technique at QM is only to mycelial or poorly-sporulating strains which are not amenable to lyophilization.

In the early development of the QM Culture Collection, a total of 2000 fungus cultures representing a wide range of tropical isolates were prepared as oil-covered slants. With the exception of a few Phycomycetes, all other isolates selected as representative of the collection remained viable for 12-24 months (1). In 1955-1956, ten years after these slants were covered, all of the cultures were checked for viability. Of the original 2000 cultures (some duplication) 669 were viable (Table I).

TABLE I*

Ten-year viability of cultures maintained under oil

		Viable	Dead	% Viable
Actinomycetales	Actinomycetaceae	4	13	24
Mucorales	Mucoraceae	10	10	50
	Mortierellaceae	0	1	0
	Piptocephalidaceae	6	2	75
	Choanephoraceae	1	38	3
Phycomycetes (Unidentified)		0	3	0
Endomycetales	Endomycetaceae	0	2	0
Eurotiales	Gymnoascaceae	2	1	67
Sphaeriales	Sphaeriaceae	53	9	85
	(Chaetomium)	(52)	(6)	
	Hypocreaceae	2	5	29
Basidiomycetes (Unidentified)		15	36	29
Moniliales	Pseudosaccharomycetaceae	4	1	80
	Moniliaceae	38	126	23
	(Gliocladium)	(1)	(44)	
	Dematiaceae	161	160	50
	(Alternaria)	(25)	(2)	
	(Cladosporium)	(47)	(16)	
	(Curvularia)	(45)	(9)	
	Stilbaceae	1	4	20
	Tuberculariaceae	92	312	23
	(Fusarium)	(82)	(285)	
Melanconiales	Melanconiaceae	5	41	11
	(Pestalotia)	(2)	(39)	
Sphaeropsidales	Sphaerioidaceae	189	159	54
	(Phoma)	(68)	(12)	
	Nectrioidaceae	2	3	40
	Leptostromataceae	0	1	0
Mycelia Sterilia		79	329	19
Unidentified		5	33	13
		669	1289	34

* Prepared by Miss Dorothy I. Fennell.

Because of its simplicity, the oil-cover technique will remain useful for relatively small general collections. We do not yet have a good substitute for moderately long-term conservation of non-sporulating isolates. Special attention in any case must be given

to the quality of the oil, to its initial sterility and dryness, to the life-expectancy of individual strains under these conditions, and to proper storage temperature for particularly delicate organisms.

With a limited staff and a large collection, periodic transfer, lyophilization, and oil-cover techniques are the most generally useful. Two other techniques, still under observation and development, eventually may replace or supplement the older standard methods.

Storage of cultures at -18° to -20°C ("deep freeze storage") is gaining attention. One general collection of 400 cultures has been stored thus for nine months on Sabouraud's agar in screw-cap tubes (2). In general, viability was maintained well through this period for a great variety of species, including strains of the usually cold-sensitive Choanephora as well as numerous isolates of dermatophytes. A second more extensive report is based on storage of 451 isolates for five years on PDA in screw-cap tubes (11). A total of 331 isolates (228 species) remained viable, these including a wide range of saprophytes and dermatophytes. All but 55 isolates, which included several of Curvularia and Helminthosporium, survived for at least two years in frozen storage. The results indicate that, after testing the survival capacity of individual isolates, deep-freeze storage of a broad spectrum of fungus cultures is a reliable procedure if new transfers are prepared every two years.

The most recent extension of preservation by freezing involves maintaining sealed vials of mycelium and spores suspended in 10% glycerol at "ultra-low" temperatures, specifically at -184.5°C in a refrigerator cooled by liquid N_2 (9). The organisms chosen for this study were ones which previously had not survived lyophilization. Included were 12 strains of Rhizoctonia, Botrytis, Pythium, Phytophthora, Syzygites, and Choanephora. All survived this drastic treatment for three days. It remains to be seen whether or not this method can be applied to a wide range of species and whether it will permit retention of viability over long periods of time.

Many laboratories, primarily industrial ones, at one time or another have used some method of preserving spore populations in sterilized soil or sand. The method has proved particularly acceptable for maintaining strains of Fusarium (15) and other equally mutable organisms (10; 3) in a stable genetic condition. With the advent of freeze-dry procedures, however, maintenance of cultures in sterile soil would appear to have lost most of its advantages, with the one exception of serving as a ready supply of abundant uniform inoculum over long periods of time.

Conservation of fungus strains on plant parts, especially leaves, is a classic technique for plant pathogens such as the

Uredinales. A report of the continued isolation of Helminthosporium sativum from wheat seed after 17 years of storage (19) is remarkable and suggestive for development, particularly as species of parasitic Helminthosporium have a poor history of survival in lyophil or under deep-freeze conditions (11). Successful establishment of a Rhizoctonia in seeds of 15 varieties of grains and of Helminthosporium carbonum in wheat seed is leading to attempts to extend this technique to numerous other fungi (4). If completely non-infested seed stocks can be obtained, maintenance of pure cultures in the form of artificially infected seeds will be of high value.

Taxonomic and Informational Responsibilities

Some microbiologists are interested only in what a specific fungus strain can do for them. Its Latin name is a bothersome anachronism and its strain number is useful primarily for listing in published tables and in requesting replacement of contaminated cultures. But such workers unwittingly close the door on one of their most potent tools, namely, the published literature to which the key is proper identification of the pertinent micro-organisms. In our time one mutant of one strain of one isolate of one fungus species may have achieved fantastic research and industrial importance; this living entity then becomes a precisely engineered tool with a special usefulness just so long as it retains its biosynthetic ability.

On the other hand, the microbiologist who is looking for a better or more versatile tool for biochemical studies or for industrial yields instantly recognizes the utility of a carefully classified organism. The taxonomy of the species immediately suggests to him related strains or species worthy of biochemical investigation. F. H. Stodola, in praising his mycological colleagues at NRRL, has commented (20): "the present high yields of penicillin, riboflavin, dextran, vitamin B_{12} and the carotenoids can be traced directly to suggestions from taxonomists that certain taxonomic groups ought to be good places to look for the desired organisms."

The point is this: certainly, as specialists in the handling of fungus cultures, we must do everything possible to insure the purity, the morphological and biochemical stability, and the availability of our isolates throughout the years; but just as certainly, we must assume with a good will the triple responsibilities (1) of identifying our own isolates with all possible precision, (2) of checking the identifications of isolates we accept from others, and (3) of contradicting shoddy or irresponsible cultural and taxonomic work. To do less than this is to bring confusion into the scientific disciplines that use our cultures and to invite ill-repute to ourselves.

QM interests in cultures, conservation, and classification have been extended in recent years to the development of a

National Index of Fungus Cultures for isolates maintained in laboratories throughout the United States. It is a common observation that over the years, as one microbiological problem after another yields pertinent data, a mass of information (often unpublished) accumulates on specific strains. Such data remain of restricted interest and of little use to anyone except the person who developed them. This situation exists in hundreds of culture collections, large and small, specialized and general. As the Microbiological Era has developed during the past 20 years, more and more often the following questions are being asked: is there a fungus strain which can perform such-and-such a biological task; and where can I obtain such an organism? If such an isolate exists in one of the large collections of the world, we are likely to know about it. But if such a strain is held in a small university or other research laboratory, the fact may escape us for years.

The National Index of Fungus Cultures was organized in our laboratory in 1955 to develop as a centralized source of as much information as can be extricated from the records of cooperating collections in the U.S. The bulk of the data centers around biological characteristics and the special usefulness of specific isolates. Additional information includes identity, authenticity, pathogenicity, special culture requirements, viability in culture, and isolation history. Each item of information, suitably coded, enters an electronically operated punched-card system. The N.I.F.C. then functions on request as a source of specialized information on the characteristics, location, and availability of these cultures. The program, although of special interest to our laboratories, has been designed and will function without restriction in the service of all microbiological disciplines.

References

1. Buell, C.B. and Weston, W.H. Application of the mineral oil conservation method to maintaining collections of fungous cultures. Am. Jour. Bot. 34: 555-561. 1947.
2. Carmichael, J.W. Frozen storage for stock cultures of fungi. Mycologia 48: 378-381. 1956.
3. Ciferri, R. and Redaelli, R. Mancata formazione di forme ascofore e conservazione di culture de funghi patogeni in substrati naturali. Mycopath. 4: 131-136. 1948.
4. Crosier, W.F. Storage of disease fungi in seeds. Farm Research 27: 10. 1961.
5. Dade, H.A. Laboratory methods in use in the Culture Collection, C.M.I., in Herb.I.M.I. Handbook. Commonwealth Mycological Institute, Kew. 1960.
6. Fennell, D.I. Conservation of fungous cultures. Bot. Rev. 26: 79-141. 1960.

7. Fennell, D. I., Raper, K. B. and Flickinger, M. H. Further investigations on the preservation of mold cultures. Mycologia 42: 135-147. 1950.
8. Hesseltine, C. W., Bradle, B. J. and Benjamin, C. R. Further investigations on the preservation of molds. Mycologia 52(1960): 762-774. 1961.
9. Hwang, S. W. Effects of ultra-low temperatures on the viability of selected fungus strains. Mycologia 52(1960):527-529. 1961.
10. Jones, K. L. Further notes on variation in certain saprophytic actinomycetes. Jour. Bact. 51: 211-216. 1946.
11. Kramer, C. L. and Mix, A. J. Deep freeze storage of fungus cultures. Trans. Kans. Acad. Sci. 60: 58-64. 1957.
12. Langeron, M. Précis de Mycologie. Masson & Cie, Paris. 1945.
13. Lukens, R. J. Conidial production from filter paper cultures of Helminthosporium vagans and Alternaria solani. Phytopath. 50: 867-868. 1960.
14. Mehrotra, B. S. and Hesseltine, C. W. Further evaluation of the lyophil process for the preservation of Aspergilli and Penicillia. Appl. Microbiol. 6: 179-183. 1958.
15. Miller, J. J. Cultural and taxonomic studies on certain Fusaria. I. Mutation in culture. Canad. Jour. Res. C. 24: 188-212. 1946.
16. Miller, P. M. V-8 juice agar as a general-purpose medium for fungi and bacteria. Phytopath. 45: 461-462. 1955.
17. Raper, K. B. and Alexander, D. F. Preservation of molds by the lyophil process. Mycologia 37: 499-525. 1945.
18. Rudman, P. (Letter of information re this Conference; C.S.I.R.O., Melbourne; August 1961.)
19. Russell, R. C. Longevity studies with wheat seed and certain seedborne fungi. Canad. Jour. Pl. Sci. 38: 29-33. 1958.
20. Stodola, F. H. The value of taxonomy and culture collections in fermentation research. (Comments at Pasteur Fermentation Cent.; New York, November 1957.)
21. Thom, C. and Raper, K. B. A manual of the Aspergilli. Williams & Wilkins Co., Baltimore. 1945.